Characterization of the Estrogen Receptor-Alpha S118P Variant in Breast Cancer

by

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Abstract

The estrogen receptor-alpha (ER) is involved with the pathogenesis and therapy of ER-positive (ER+) breast cancers. ER signaling is complex, though many mediators of ER signaling have been identified. Specifically, phosphorylation of ER at serine 118 affects responses to estrogen and therapeutic ligands, and has been correlated with clinical outcomes in ER+ breast cancer patients. We have identified a novel, naturally-occurring germline variant by which ER serine 118 is replaced with a proline residue (S118P). We have developed and characterized human isogenic breast epithelial cell line models carrying the ER S118P variant to determine the functional effects of this variant. Surprisingly, in heterozygous knock-in models, S118P cells demonstrate no significant change in proliferation, migration, signaling, or response to the endocrine therapies tamoxifen and fulvestrant. We also examined the frequency of this variant in families at high risk for breast cancer and concluded that it is not enriched in this population. This work suggests that ER S118P does not affect the risk of breast cancer incidence, and importantly, breast cancer patients harboring this variant are not likely to have altered responses to current endocrine therapies.

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Table of Contents

List of Tables

List of Figures

1 Introduction

Breast cancer background

Breast cancer is currently the most-diagnosed cancer in women in the US, with over 250,000 new cases expected annually. Over 40,000 deaths per year in the US are attributed to breast cancer, and there are millions of survivors being treated and monitored throughout the country at any one time.

Breast cancer is a disease of the epithelial cells which line the ducts and lobules of breast tissue. Genetic alterations in these cells can result in cell cycle dysregulation and uncontrolled cell growth. Under the proper conditions, these cells may establish a tumor and eventually breach the epithelial basal membrane, becoming a bona fide invasive carcinoma.

Breast cancers are often classified according to several pathological factors upon diagnosis: whether the tumor cells originated in the ducts or lobules of the breast;

whether they derive from basal- or luminal-type epithelial cells; and the expression of three key receptors on the cell surface—the estrogen receptor-α (ER), the progesterone receptor (PR), and the human epidermal growth factor receptor 2 (HER2).

ER is expressed in approximately 70% of breast cancers, usually in tandem with other markers that are often found in luminal breast epithelial cells; ER-expressing (ER+) breast cancers have thus been deemed luminal-type. This group has been further divided into luminal A and luminal B subtypes, distinguished by significant expression of proteins such as HER2 or Ki67 in luminal B tumors. Luminal A type breast cancer is generally associated with a better prognosis than the other breast cancer subtypes due to a combination of factors: it tends to be more differentiated and less aggressive than other subtypes, the rate of recurrence is lower and slower, and there are ever-increasing numbers of targeted therapies against ER and ER-related signaling being developed and approved for treatment of this disease subtype. Generally speaking, most but not all luminal A type breast cancers are ER+ and HER2- for receptor expression.

However, the positive outlook associated with ER+ breast cancer turns out to be relatively short term. ER+ breast cancer patients who have been treated with standard of care targeted therapies continue to have significant rates of recurrence over twenty years post-diagnosis (1). The fact that ER+ patients continue to experience recurrence of disease over such a long period provides a rationale for continuing to develop and optimize therapies for this very large population of breast cancer survivors.

The estrogen receptor-alpha and estrogen signaling

The ER protein, encoded by the gene *ESR1*, is a nuclear steroid hormone receptor which acts as a transcription factor to regulate proliferation and differentiation of cells in which it is expressed. Estrogen signaling via ER plays a major role in elements of the female reproductive cycle such as ovulation and mammary gland development, and ER is highly expressed in corresponding tissues such as the ovaries and breasts and has been linked to carcinogenesis in those tissues.

The approximately 70% of breast cancers which express ER are dependent on estrogen signaling for tumor growth and maintenance. In "classical" ER signaling, binding of the ligand estrogen to ER induces dimerization to form an activated transcription factor, which can then regulate transcription of many downstream genes (Figure 1). In addition to a ligand-binding domain (LBD), ER contains a DNA-binding domain which recognizes estrogen response elements (EREs) in the genome and a transactivation domain which induces changes in transcriptional activity at EREs upon ligand-dependent receptor activation and DNA binding. A number of co-activators and co-repressors have been shown to form complexes with ER in order to modulate its activity—the balance of co-activator/co-repressor expression in different tissues and under different cellular conditions is thought to be a major factor in ER functionality and even tissue-specific responses to endocrine therapies targeting ER signaling in the cancer setting.(2, 3)

In "non-classical" ER signaling, ER can alternatively interact with non-ERE genomic loci and/or become activated under ligand-independent conditions, and

participates in crosstalk with several other major signaling pathways in the cell, including the MAP Kinase and PI3 Kinase pathways (4, 5). Transcription factors such as AP-1, Sp1, and NFKB can interact with ER, conferring the ability to recognize non-traditional response elements for transcriptional activation (6). Interactions with coactivators and corepressors can also affect ER's cellular location, responsiveness to ligand, and transcriptional activity (7, 8). Additionally, post-translational modifications to ER that occur downstream of several receptor tyrosine kinases and G-protein coupled receptors can induce ligand-independent ER signaling. This includes modulating three well-characterized phosphorylation sites of ER which can be acted upon by important additional kinases such as MAP kinase, Akt, protein kinase A, and HER2 respectively (9, 10). The integration of these well-described pathways with ER activity has profound implications for normal ER function, as well as its role in cancer and resistance to endocrine therapies. For example, phosphorylation of ER by HER2 and other kinases may circumvent estrogen-dependence of ER activation, contributing to resistance to anti-estrogen endocrine therapies (11). ER is also capable of non-genomic signaling activities, and has been described to have non-nuclear activity via interactions with a number of important cellular signaling factors, most notably proteins within the MAP kinase signaling pathway (12).

Endocrine therapies

The difficult-to-tolerate side effects of systemic chemotherapy have led to efforts to identify and develop targeted cancer therapies wherever possible. The crucial

role of ER in luminal breast cancer has led to widespread use of three broad classes of endocrine therapies against ER signaling in the breast (Figure 2).

A class of drugs deemed selective estrogen receptor modulators (SERMs) confers a change in ER activity based on conformational change and/or co-repressor binding. All SERMs are partial ER agonists, exhibiting anti-proliferative activity in breast tissue but estrogenic activity in other peripheral tissues such as endometrium and bone, depending on the particular SERM (13). In randomized clinical trials, the first-generation SERM tamoxifen has performed well and subsequently been approved for prevention of breast cancer in high-risk patients, as an adjuvant treatment for early disease in pre- and postmenopausal women, and as first-line therapy in metastatic breast cancer. However, tamoxifen use is associated with increased risk of endometrial cancer and thromboembolic events. Raloxifene, a second-generation SERM, is also used for prevention of breast cancer and has shown a reduction of side effects, notably endometrial cancer, when compared with tamoxifen in breast cancer clinical trials (although raloxifene's primary use is in the treatment of osteoporosis) (13). Importantly, raloxifene is not used for the treatment of breast cancer. Several third-generation SERMs are currently under investigation in clinical trials, including toremifene, which has been used successfully in the early adjuvant and metastatic settings but has a safety profile similar to that of tamoxifen and bazedoxifene.

Selective estrogen receptor down-regulators (SERDs) such as fulvestrant reduce ER activity and induce its proteasomal degradation. Unlike SERMs, fulvestrant and other SERDs exhibit full antagonistic activity of ER in all tissues. Fulvestrant is currently a

second-line endocrine therapy given to postmenopausal ER+ breast cancer patients with advanced disease, and promising results from recent clinical trials indicate that it may be used in other settings as well, such as first-line therapy and in premenopausal patients (14, 15). In addition to fulvestrant, two candidate SERDs with improved bioavailability are being evaluated in Phase I clinical trials (16, 17). These latter two SERDs have the significant advantage of oral formulations, compared to fulvestrant which requires monthly gluteal intramuscular injections.

The final class of ER-targeted endocrine therapies, the aromatase inhibitors (AIs), greatly reduce production of estrogen in the body, thus removing the main source of ER activation. This class includes the steroidal AI exemestane, as well as the non-steroidal AIs letrozole and anastrazole. AIs can only be used in post-menopausal women or pre-menopausal women who no longer have ovarian production of estrogen, either through surgical removal of the ovaries or from lutenizing releasing hormone agonists, such as goserelin. This is because ovarian production of estrogen does not rely upon aromatase, an enzyme that is a cytochrome P450 family member. In the post-menopausal state, aromatase is the major if not exclusive source of estrogen production, and AIs for these patients have been proven to be superior to tamoxifen in the majority of trials. Thus, these drugs constitute the standard first-line adjuvant and metastatic endocrine therapies for post-menopausal ER+/HER2- breast cancer patients.

Endocrine therapy resistance

Although all three drug classes are used effectively in patients with ER+/HER2- breast cancer, resistance to these therapies has become a major hurdle (11). Almost half of ER+/HER2- patients do not respond to endocrine therapies, termed primary or *de novo* resistance. In addition, the majority of metastatic patients who initially respond to endocrine therapies will eventually progress or relapse, termed secondary or acquired resistance. The mechanisms for resistance have not been fully elucidated, but depend upon estrogen-independent survival and proliferation of the tumor cells. This can result from ER mutations which lead to constitutive ligand-independent activation of the protein and/or activation of other mitogenic pathways which circumvent estrogenic signaling.

Combination therapies

Due to challenges with resistance to endocrine therapies, additional options for the treatment of ER+/HER2- breast cancer have emerged and others are under investigation. As in other cancers, it is thought that combination therapies, by halting multiple signaling pathways at once, will prevent ER+/HER2- tumors from evolving compensatory mechanisms to bypass ER signaling. Therefore, a number of non-endocrine targeted agents have been tested in combination with endocrine therapies and more studies are underway. Several promising agents have been identified and are now approved therapies for ER+/HER2- disease.

Complex crosstalk between PI3 kinase/mTOR and ER signaling pathways suggests that targeting of both pathways could be detrimental to ER+ tumor growth (18). For this reason, the BOLERO trials tested the hypothesis that the mTOR inhibitor everolimus in combination with current endocrine therapies could improve outcomes for metastatic ER+ patients with endocrine therapy resistant disease. Improved outcomes demonstrated by the BOLERO-2 trial have resulted in approval of the combination of everolimus and exemestane for clinical use in advanced ER+ breast cancers (19). Further BOLERO studies will test everolimus in combination with other endocrine agents such as letrozole and in additional breast cancer settings such as premenopausal and/or endocrine-naïve patients (20).

Another hypothesis posits that cell cycle arrest could enhance endocrine therapy (21). The CDK 4/6 inhibitors palbociclib and ribociclib are therefore interesting candidates for combination therapy, and numerous trials have been initiated with these agents. Early results of the PALOMA trials have demonstrated improved outcomes with palbociclib in combination with certain endocrine therapies including letrozole and fulvestrant and further studies are underway (20, 22). This has led to the FDA approval of letrozole and palbociclib for first line therapy in metastatic ER+/HER2- breast cancer patients.

Finally, HDAC inhibitors such as entinostat may deregulate *ESR1* transcription sufficiently to overcome endocrine resistance. The ENCORE 301 study demonstrated improved outcomes with entinostat and exemestane combination therapy and confirmatory trials are underway (19).

On the other hand, studies of several additional classes and combinations of targeted agents have met with limited success in ER+ breast cancers. Anti-angiogenic agents, multi-target kinase inhibitors, EGFR inhibitors, and poly-endocrine therapy did not significantly improve outcomes in their respective trials but may benefit from the identification of biomarkers that can select for patient populations most likely to benefit from these therapies either singly or in combination.

Amplification of ESR1

Although ER is highly expressed in a large proportion of breast cancers, it appears that this is largely not a product of *ESR1* amplification. This issue was controversial after a 2007 fluorescent in situ hybridization study indicated that significant *ESR1* amplification was present in over 20% of breast cancers (23). Later studies failed to replicate this rate of amplification and it was eventually suggested that accumulation of *ESR1* transcripts in the nucleus may have been responsible for the perceived amplification and its association with strongly ER+ breast cancer (24). However, another recent study found that amplification of *ESR1* occurred in MCF7 cells (a widely used breast cancer cell line) after long-term estrogen deprivation, which may model the paradoxical clinical phenomenon in which long-term endocrine therapy in a small subset of patients leads to estradiol hypersensitivity (25). In this group, estradiol may be useful as a breast cancer therapy which causes apoptosis in hypersensitive cells, thus stabilizing the tumor.

ER variants

With the understanding that all cancers are genetic diseases, it is surprising that genetic alterations in *ESR1* are rarely found in ER-driven primary breast tumors. Early *in vitro* mutagenesis studies of *ESR1* identified dominant-negative and ligand-independent forms of the ER protein (26) and *in vivo* and clinical studies identified acquired mutations which mediate the effects of tamoxifen (27, 28), but large clinical surveys have shown that ER variants are rare in primary breast cancer (29). This knowledge from early tumor-sequencing studies led to the notion that *ESR1* gene alterations are not involved with breast carcinogenesis despite the importance of ER signaling for therapeutic intervention in the majority of breast cancer patients.

However, it has been suggested that ER variants play a role in advanced breast cancer (30) and indeed, contemporary studies have identified rearrangements and recurrent somatic mutations of *ESR1* in metastatic hormone-resistant disease (25, 31-34). Each of these studies has detected a significant rate of *ESR1* mutation or variation (Table 1). In various forms, the authors of these studies suggest that the low-estrogen conditions achieved by the endocrine therapies favor somatic variation of *ESR1* in order for tumor cells to adapt and thrive, and these mutations are now known to be mediators of resistance to endocrine therapies. Thus, although the known *ESR1* mutations are not likely to be involved with the development of breast cancers, the recognition of *ESR1* mutations in metastatic disease may lead to newer therapies that can overcome resistance for therapeutic benefit.

Notably, there appears to be a mutational hot spot region in the ER LBD at residues 536-538, which includes a tyrosine phosphorylation site at residue 537 (Figure 3). Mutagenesis studies have identified the critical role of these amino acids in regulation of ER activity (35, 36). A number of different substitutions within this hot spot have been shown to confer constitutive ligand-independent activation of ER, suggesting that these naturally-occurring mutations play a role in acquired resistance to endocrine therapies (25, 31-34). These residues lie within helix 12 of the LBD, which is responsible for closing the LBD pocket upon ligand binding and creating a surface with which co-regulators can interact. As shown in structural modeling of one particular LBD mutation, D538G, changes to helix 12 can result in a conformational change that, even in the absence of ligand, mimics the ligand-bound form of ER (34). This not only precludes binding of ligands such as estradiol, tamoxifen, and fulvestrant but also allows ligand-independent co-activator recruitment. Finally, this ligand-independent ER has constitutive transcriptional activity at EREs and may contribute to enhanced cell proliferation and migration (34).

A study of patient-derived breast cancer xenografts, which also detected LBD hot spot mutations, noted one chromosomal translocation between the coding regions of *ESR1* and *YAP1* leading to an in-frame, expressed fusion protein (25). Much like two previously identified naturally-occurring ER fusion proteins, the N-terminus of ER containing the DNA-binding domain and AF1 transactivation domain was preserved but the C-terminus was replaced by that of YAP1—thus losing the LBD and AF2 transactivation domain of ER. Although the resulting physical properties of this protein

are quite different from the LBD mutations discussed above, this fusion protein similarly gained ligand-independence while preserving DNA-binding and transactivation function. However, while LBD point mutations will alter ligand affinities and contribute to resistance to particular endocrine therapies, complete loss of the LBD as in these fusion proteins leads to an "intrinsic and universal endocrine-therapy resistance" which will need to be addressed with different clinical strategies (25). While clearly occurring at a low frequency, the recurrence of this type of fusion protein across three studies indicates that the effect is robust and almost certainly drives endocrine therapy resistance in this small subset of patients.

While the effects of the LBD mutations and such fusion proteins have been explored, there are still ER variants awaiting study. Less frequent missense mutations have been identified in clinical trials by several groups (37-39) and it is unknown how these variants affect ER signaling activities within the cell. One interesting variant, known as ER S118P, will be the focus of Chapter 2.

It is likely that ER variants can be categorized according to the type of endocrine therapy that they resist: SERM and SERD resistance depends upon changes to the protein's interaction with the drug, while AI resistance depends upon estrogen-independent activity of the receptor or of the cell itself. Since ER varies widely in its affinity for various ligands, it may be that each individual LBD mutation will confer a different effect on each individual receptor-ligand interaction, which will have profound effects on endocrine therapy decisions for patients with these mutations.

Finally, it cannot be ignored that these mutations were identified only after the recent practice of obtaining metastatic biopsies for sequencing. Although some studies have suggested that these LDB mutations are present in primary tumors (33), most studies have not validated these results. More importantly, we now understand based on these studies and others that a single biopsy from the primary tumor is usually not representative of a patient's heterogeneous and ever-evolving tumor cell population. For example, some of the aforementioned studies of *ESR1* mutations identified mutations in one site of disease but not in other metastatic sites within the same patient (33, 34). In this respect, we have demonstrated that a "liquid biopsy" technique, which is representative of all disease sites in the body including micrometastatic disease, can detect low-frequency mutations including those in *ESR1* (40). We speculate that in the future this will provide additional information to make the best treatment decisions. Thus, the lesson learned from cancer genome sequencing studies over the past decade is that profiling a patient's primary tumor may be inadequate to truly understand the molecular evolution of metastatic disease. In this regard, liquid biopsies may allow a more comprehensive profiling of a patient's total disease burden, and for ER positive disease, further characterization of *ESR1* variants. This, along with ongoing drug development, has brought about a positive outlook for ER+/HER2- breast cancer patients.

Rationale

The characterization of clinically relevant *ESR1* variants and their sensitivity to various endocrine therapies will be crucial for optimal treatment for this subset of ER+ breast cancers. The advent of molecular profiling and precision medicine brings the opportunity to account for these *ESR1* variants and treat patients accordingly. While new therapies may be needed for certain variants, higher doses of already approved therapies may afford near term benefit, a testable hypothesis for clinical trials.(34) In addition, the continued development of newer SERMs and SERDs, along with novel therapeutic combinations, may allow for improved outcomes for patients with metastatic ER+/HER2- disease.

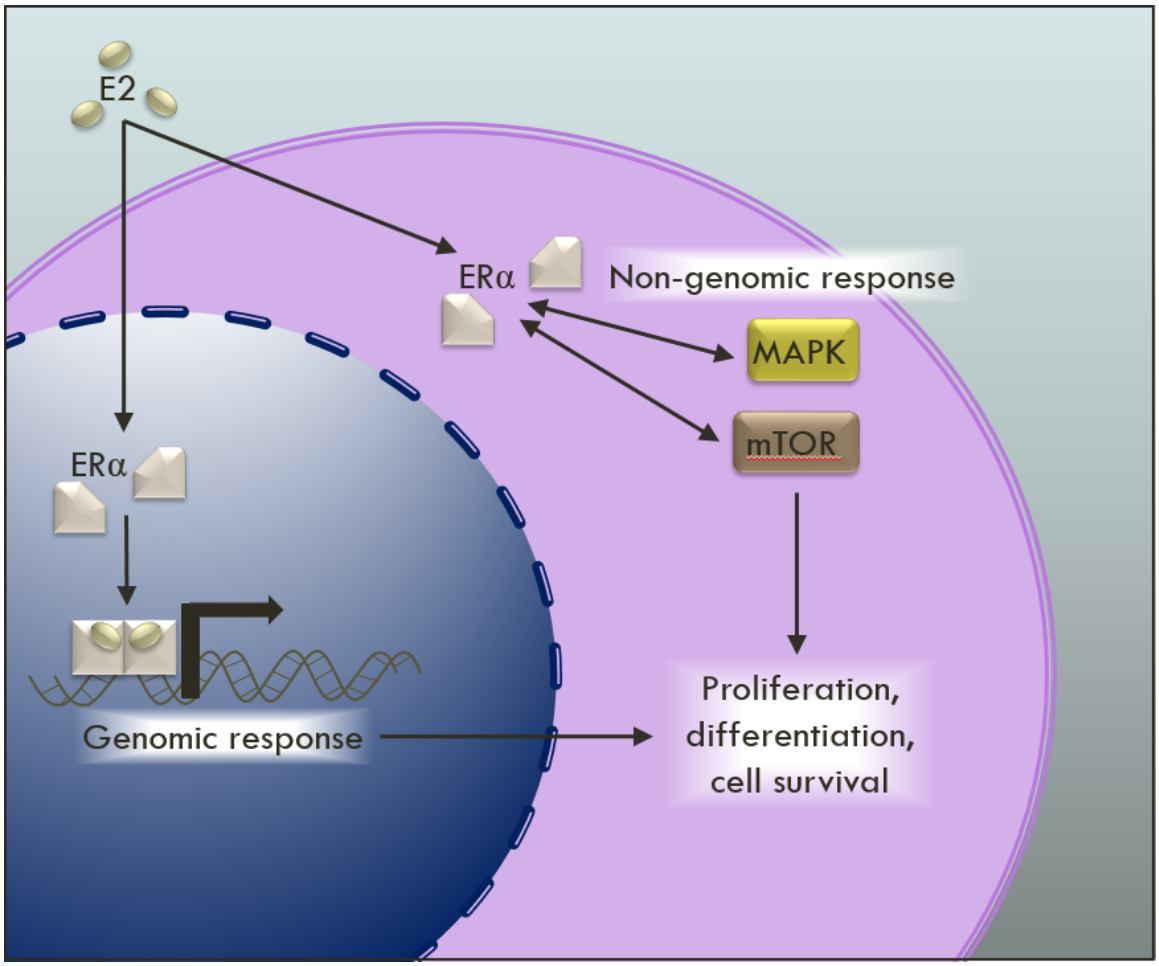

Figure 1: Estrogen and ER signaling. Estrogen (E2)-induced activation of the estrogen receptor leads to proliferation, differentiation, and cell survival. Classical ER signaling consists of a genomic or transcriptional response, and non-classical ER signaling involves non-nuclear associations with other cellular pathways.

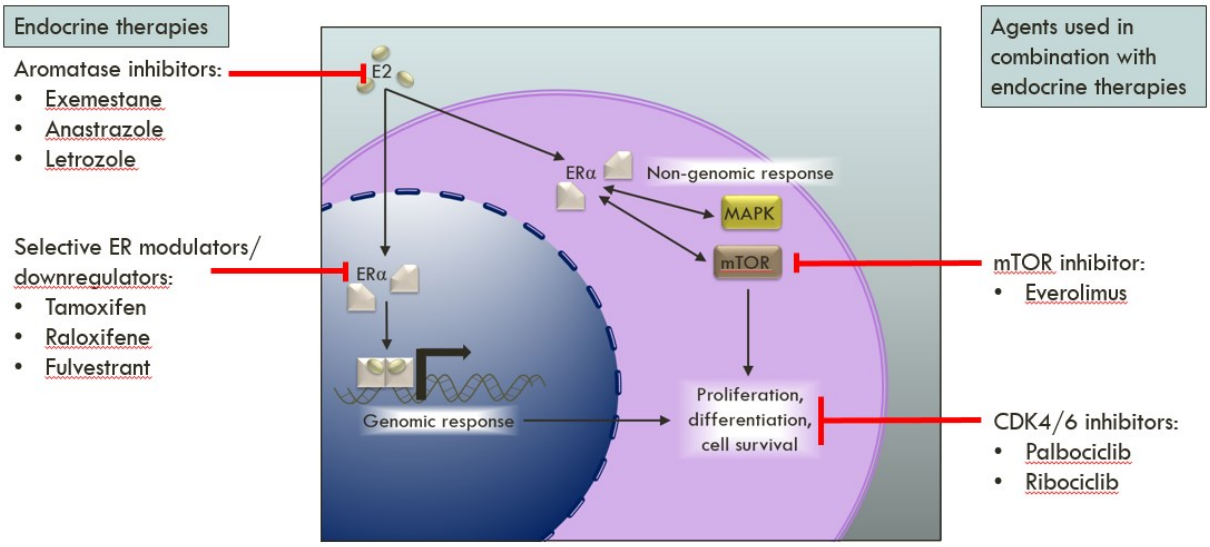

Figure 2: Targeted therapies against ER. Targeted therapies for ER+ breast cancer consist of endocrine therapies, including AIs, SERMs, and SERDs, as well as targeted agents for interacting pathways such as mTOR and CDKs.

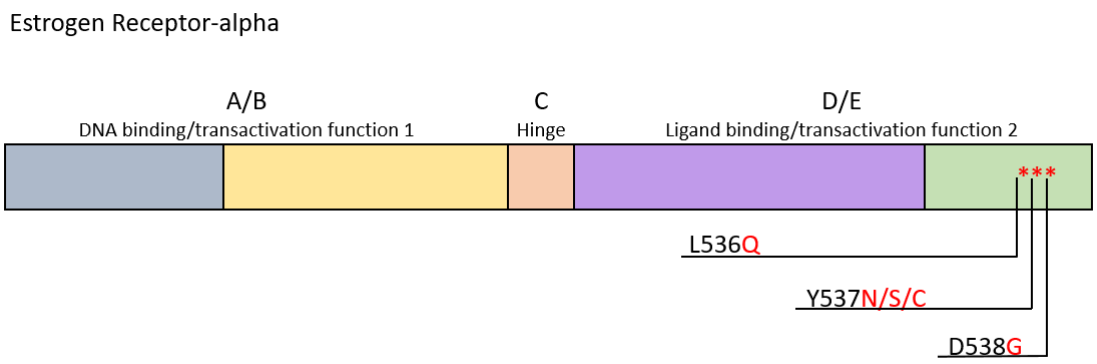

Figure 3: ER protein ligand-binding domain mutations. The gene *ESR1* encodes the nuclear receptor protein estrogen receptor-alpha, containing domains responsible for DNA binding, transactivation functions 1 and 2, and ligand binding. The most common *ESR1* mutations result in variation of the ligand binding domain at residues 536, 537, and 538. These residues are part of Helix 12 of the ligand binding domain, which is known to regulate ligand binding and recruitment of co-repressors and co-activators, therefore constituting the basis for ligand-dependent activation of the estrogen receptor-alpha.

Study	ER variant types detected	n	Variant rate
Li et al 2013	Amplification, fusion, mutation	7	57%
Merenbakh-Lamin et al 2013	Mutation	13	38%
Robinson et al 2013	Mutation	11	55%
Toy et al 2013	Mutation	80	18%
Jeselsohn et al 2014	Mutation	76	12%

Table 1: ER variant rates. In 2013 and 2014, five studies detected a significant rate of ER variation in hormone-resistant metastatic breast cancer.

2 The ER S118P variant in breast cancer

Due to the importance of ER-targeted therapies for patient care, it is crucial to identify patients whose disease may be resistant to this class of drugs. While various studies have described resistance mechanisms (41) including constitutive ER activation in metastatic disease via mutations in the ER ligand binding domain (LBD) (25, 31-34), most patients with hormone therapy-resistant ER+ disease are identified by disease progression and the molecular mediators of their resistance are unknown.

Review of next generation sequencing data from metastatic patient samples from the Johns Hopkins Molecular Tumor Board and other studies revealed several patients with an unusual *ESR1* variant (37-39). This single-nucleotide substitution results in a non-synonymous substitution of serine to proline at residue 118 of ER (ER S118P), in the region of the DNA-binding and transactivation function 1 domain of the protein. Serine 118 is an important phosphorylation site dictating ER activity levels as well as associations with coregulators and estrogen response elements (8). S118

phosphorylation has been well-characterized and is mediated by several kinases such as MAPK, mTOR, and CDK2 (42, 43). Therefore, in addition to the potential change in protein structure resulting from this substitution, it also constitutes loss of a crucial regulator of ER signaling. Phosphorylation at serine 118 has also been shown to correlate with tamoxifen resistance (44). Multiple studies have shown profound differences in ER function upon S118 mutation to alanine or glutamic acid, precluding or mimicking phosphorylation, respectively (8, 41, 45). Due to the importance of the S118 residue in ER function, we hypothesized that S118P may be involved in ER+ breast cancer pathogenesis and/or drug resistance. In this study, we created genetically altered breast epithelial cell line models in order to determine the cellular phenotypes that are associated with this variant. In addition, we queried germline DNA samples from breast cancer patients and high-risk families to evaluate the allele frequency of ER S118P in the context of breast cancer incidence; we compared this data to publically available general population data to assess enrichment of the variant in breast cancer.

Results

Isogenic targeting and overexpression of ER S118P in human breast epithelial cells

In order to study the functional consequences of ER S118P in human breast cells, we developed three model cell lines (Figure 4). First, we created isogenic knock-ins within the MCF7 and T47D cell line backgrounds. The widely used MCF7 and T47D cell lines are each derived from a metastatic breast adenocarcinoma and express endogenous ER. We used isogenic gene targeting, via a recombinant adeno-associated

viral vector containing the sequence of *ESR1* exon 1 modified with the single base-pair substitution leading to ER S118P, to produce single-cell clones heterozygous for ER S118P (Figure 5). These cell lines were designated MERSP (MCF7 ER S118P) and TERSP (T47D ER S118P) and two individually isolated clones were derived for each. We also derived a "targeted wild-type" clone for each cell line background, which underwent the same viral infection and subsequent isolation steps as the variant clones but does not carry the variant of interest. These were designated MWT and TWT, for MCF7 targeted wild-type and T47D targeted wild-type, respectively.

In addition, we used the non-tumorigenic MCF10A cell line, which was derived from fibrocystic breast tissue and does not express ER endogenously, to develop an overexpression model of the ER S118P variant. Our lab has previously established an overexpression model of wild-type ER in this cell line, designated ERIN (ER in nontumorigenic) (46). For this study we transfected an isogenic plasmid containing a copy of ER S118P cDNA to create two mERIN (mutant ER in nontumorigenic) cell line clones (Figure 6).

To confirm integration and stable expression of the variant protein in our isogenic knock-in models, we performed DNA and cDNA analysis by droplet digital PCR and Sanger sequencing as well as protein analysis by Western blot. All MERSP and TERSP clones demonstrate integration of the variant construct within the endogenous locus and expression of the variant at the mRNA level via cDNA sequencing (Figure 7). One caveat to these models is that genomic DNA analysis suggests that off-target incorporation of the viral construct may have occurred in MERSP1, MERSP2, and TERSP2

as indicated by higher levels of the variant allele, however it does not appear that any additional copies outside of the endogenous locus are expressed since variant cDNA levels are similar or slightly lower as compared to wild-type (Figure 8). In the mERIN overexpression model, we confirmed high levels of expression of the variant mRNA and protein which are comparable to wild-type ER levels in the ERIN cell lines (Figure 9).

Cells expressing ER S118P grow similarly to wild-type controls

To determine whether cells expressing ER S118P have a growth advantage over parental cells and whether they respond to estrogen exposure, we compared cell proliferation over one to two weeks. MERSP1 and MERSP2 grew similarly to their parental and targeted wild-type counterparts, both in the presence of 1 nM 17-beta estradiol (E2) and vehicle control (Figure 10). TERSP2 appears to proliferate more rapidly than its parental and targeted wild-type cell lines, while TERSP1 appears to proliferate more slowly; while both clones respond to 1 nM E2, the difference in their baseline growth rates remains obvious even in the presence of E2 (Figure 11). This discrepancy suggests that other changes within these clonal T47D cell lines are responsible for the difference in rate of proliferation, since it is unlikely that this isogenic change would produce two opposite responses in the absence of other genetic factors.

In our overexpression model, it appears that the mERIN clones grow similarly to the wild-type ER containing line ERIN7 in the absence of estrogen, but do not respond to 100 nM E2 as strongly as ERIN7 (Figure 12). It seemed that these clones, especially mERIN7, actually had a decreased rate of proliferation as compared to baseline growth

in the absence of E2, so we explored additional E2 concentrations in order to determine whether the optimal E2 dose for these cell lines may be lower than that of ERIN7 (Figure 13). Indeed, at 1 nM and 10 nM E2 mERIN7 displays a subtle increase in proliferation which is not present at higher or lower E2 concentrations. mERIN9 responds strongly to 10 nM E2 and higher concentrations.

Overall, it appears that ER S118P does not confer a measurable change in cell proliferation at baseline, and the presence of one copy of wild-type ER in our heterozygous models is enough to drive a standard estrogen response. In the absence of any wild-type ER, as in our overexpression model, the cells do not respond as strongly to estrogen indicating that the variant ER protein may not be as highly activated by its traditional ligand.

The ER signaling pathway is intact in ER S118P-expressing cells

In light of ER's classical role as a transcription factor and the phosphorylation of S118 as a dynamic effector of ER transcriptional regulation, we wanted to assess E2-induced expression of a set of genes known to be regulated by ER. Across a panel of 90 genes in the ER signaling pathway, overall expression patterns with vehicle or 10 nM E2 treatment were consistent between MCF7 and MERSP1 cells (Table 2). Therefore we conclude that there is no significant change in ER-related gene expression pattern between the two cell lines, indicating that the networks of transcriptional regulation in which ER participates are widely unaffected by the presence of the ER S118P variant as a single copy.

The MAP kinase signaling pathway is known to interact heavily with ER signaling and is crucial to the overall behavior of a cell. In order to determine if the ER S118P variant affects the activity of this pathway, we probed for levels of phosphorylated Erk to represent pathway activation. After 24 hours of exposure to E2, p-Erk activation is relatively consistent across all cell lines and is not associated with expression of ER S118P (Figure 14).

Cells expressing ER S118P remain sensitive to hormonal therapies and retain cancerous phenotypes similar to wild type ER cells

With the clinical value of this variant in mind, we sought to determine whether our models would respond to hormone therapies commonly used in patients with ER+ disease. Tamoxifen and fulvestrant both interact directly with ER to reduce estrogen signaling in breast cancer cells, and other ER mutations have been shown to mediate resistance to these therapies (25, 31, 32). However, none of the ER S118P knock-in cell lines displayed any significant differences in response to tamoxifen or fulvestrant at various concentrations over nine days of drug treatment (Figure 15). This response to endocrine therapies suggests that ER S118P does not interfere with ER protein-drug interaction.

We were also interested in the migratory abilities of ER S118P cells due to recent studies which suggest that ER LBD mutations at Y537 and D538 can increase the metastatic potential of cells (47). In order to determine if the S118P variant could similarly contribute to a change in the migratory properties of cells, suggesting a change

in metastatic potential, we performed a scratch wound healing assay on the MCF7 cell line and its ER S118P derivatives. In the absence and presence of E2, there was no consistent difference in wound closure between cells expressing ER S118P and their wild-type counterparts (Figure 16).

LBD ER mutations also constitutively activate ER leading to E2 independent growth in vitro and in vivo (25, 31, 32). Accordingly, we assessed the ability of the S118P variant cells to form tumors *in vivo*. Parental MCF7 and MERSP1 cells were injected into the mammary fat pad of athymic nude mice, and tumor growth was monitored over seven weeks. As previously described, MCF7 cells were only able to form tumors with exogenous estrogen supplementation. Similarly, MERSP1 cells were non-tumorigenic in the absence of estrogen but formed large, rapidly-progressing tumors in the presence of estrogen (Figure 17). The size and growth rate of resulting tumors were similar between the parental and variant cell lines. T47D and MCF-10A cell lines could not be assessed due to their inability to grow in athymic nude mice.

The ER S118P allele frequency is comparable between the general population and families at high risk for breast cancer

We and others first noted the ER S118P variant from commercial NGS assays that did not employ germline filtering, with the suggestion that this variant was somatic. Further analysis determined that ER S118P was a germline variant in at least one patient, and during the course of our study the variant was added to dbSNP under the identifier rs200075329, with the clinical significance of this substitution listed as “NA”. In addition

to our functional characterization, we sought to determine the frequency of this variant in a population at high risk for breast cancer based upon family history and the absence of germline BRCA1 and 2 variants, including pathogenic and variants of unknown significance (VUS). As a missense variant at a highly functional residue in the ER protein, we hypothesized that ER S118P may be correlated with either a higher or lower breast cancer incidence, due to a change in the signaling pathways that can lead to breast cancer development when dysregulated.

The minor allele frequency of rs200075329, corresponding to the substitution ER S118P, varies from 0.0016 to 0.0098 in the general population according to various exome sequencing studies (Table 3). In a cohort of 554 individuals with a family history of breast cancer, consisting of 277 breast cancer patients and 277 age-matched controls, we identified four heterozygous carriers of the ER S118P variant (Table 4). In our cohort, the minor allele frequency of this variant was 0.004. Based on these data, the variant is not altered in a population of individuals with a high risk of genetic drivers of breast cancer. In addition, of the four carriers of the variant, two were breast cancer patients and two were age-matched controls, indicating that presence of the variant is not correlated with disease incidence (Table 5).

Discussion

The importance of serine 118 phosphorylation in cellular ER dynamics and hormone response has been described in numerous studies. ER is involved in complex transcriptional regulation networks and S118 phosphorylation has effects on its

coregulatory binding partners, activation time course, and response to ligand binding (10). Phosphorylated ER S118 has also been shown to feed directly back onto levels of ER in the cell via changes in degradation dynamics as well as binding to the *ESR1* promoter (48).

In prior research, cells expressing a variant of ER with an alanine at residue 118 (S118A) have displayed significant changes in transcriptional regulation activities, estrogen-induced growth stimulation, and response to tamoxifen (8, 41, 49). Disruption of ER phosphorylation at S118 by shRNA has also been shown to confer marked phenotypic changes in a breast cancer cell model (50). To our knowledge, ER S118 mutants have been studied only in overexpression constructs, meaning they have not been under control of the endogenous promotor or had the capacity to interact with wild-type ER protein. We hypothesized that heterozygous knock-in of a naturally-occurring ER variant, ER S118P, would drive similar changes and so we developed breast cancer cell line models containing the variant of interest. However, the present studies have shown that single copy ER S118P is not sufficient to alter proliferation, migration, or sensitivity to tamoxifen and fulvestrant of breast cancer cells *in vitro*. In addition, we do not see significant changes in expression of ER-regulated genes or phosphorylation of AKT, all of which are known to be affected by ER S118 phosphorylation dynamics. Our data suggest a slight upregulation of the Erk pathway in response to E2 in heterozygous ER S118P cells, but this molecular change does not lead to a detectable cellular phenotype in our assays. Finally, these cells behave similarly to their wild-type counterparts as estrogen-dependent xenografts in mice.

We have not found any evidence that this ER variant contributes to a significant cellular or molecular phenotype in breast cancer cells according to the heterozygous models we have characterized. Our current hypothesis is that the remaining wild-type ER protein in heterozygous models is sufficient to carry out approximately normal estrogen signaling in the cell. This is surprising since ER dimerizes upon activation and the majority of ER dimers in a heterozygous cell would contain at least one variant unit. However, if the protein structure is not significantly changed by the variant, and phosphorylation of only one subunit is sufficient for the regulation of signaling, then it is possible that the majority of dimers would function normally without obvious phenotypic changes.

In addition, we have analyzed a population of individuals diagnosed with and at risk for breast cancer based on family history of disease. We hypothesized that this variant may be involved in cellular signaling changes which could be associated with the development of breast cancer, and therefore that the variant may have an altered prevalence in a population representing familial breast cancer and its genetic drivers. However, the variant was present at a minor allele frequency of 0.004, consistent with the average range of minor allele frequencies identified in the general population. This result suggests that ER S118P is unlikely to be a modifier of breast cancer development.

In light of these results, this variant may be benign in breast cancer patients. It does not appear to function as a driver or supressor of any particular characteristics of the cancer which we have studied. Therefore, we recommend that patients who are

found to carry this variant should be treated according to standard of care, since this variant does not seem to alter breast cancer incidence or response to therapies.

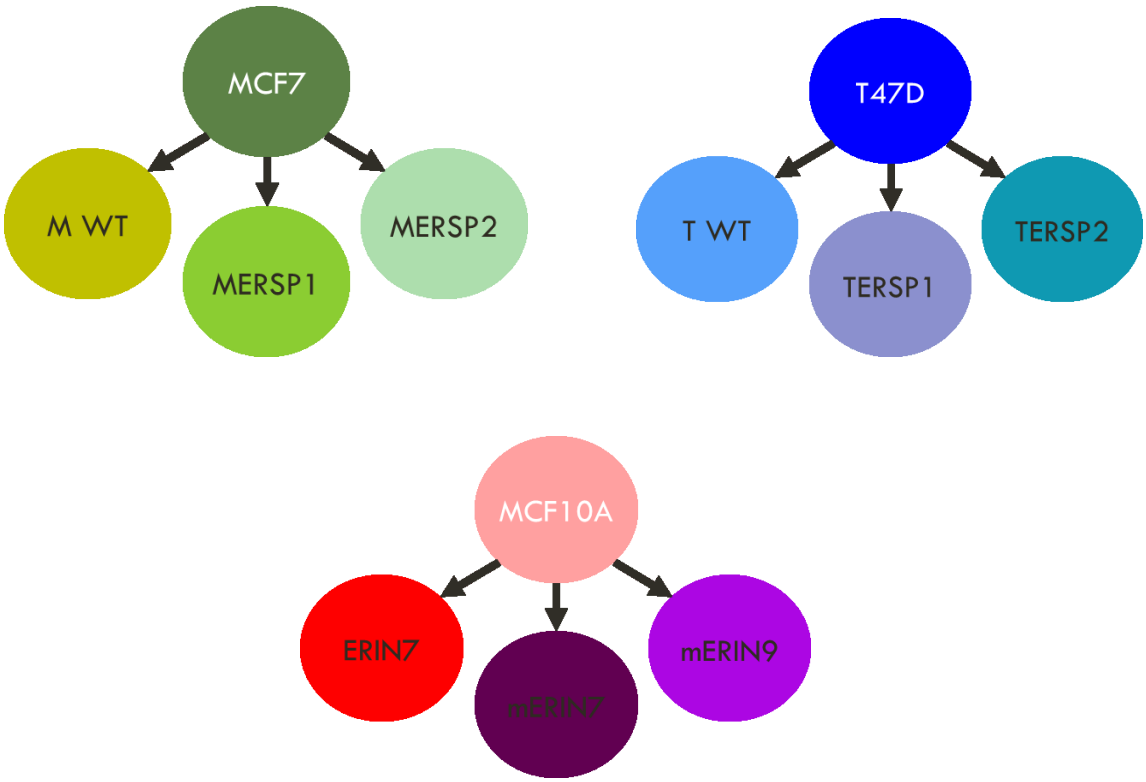

Figure 4: ER S118P cell line panel schematic. From each parental cell line used in this study, three additional cell line derivatives were obtained. MERSP1, MERSP2, TERSP1, and TERSP2 express heterozygous ER S118P while MWT and TWT are homozygous WT at that locus but underwent the same viral targeting and screening process. mERIN7 and mERIN9 exogenously express ER S118P while ERIN7 exogenously expresses WT ER via the same pIRESneo vector backbone.

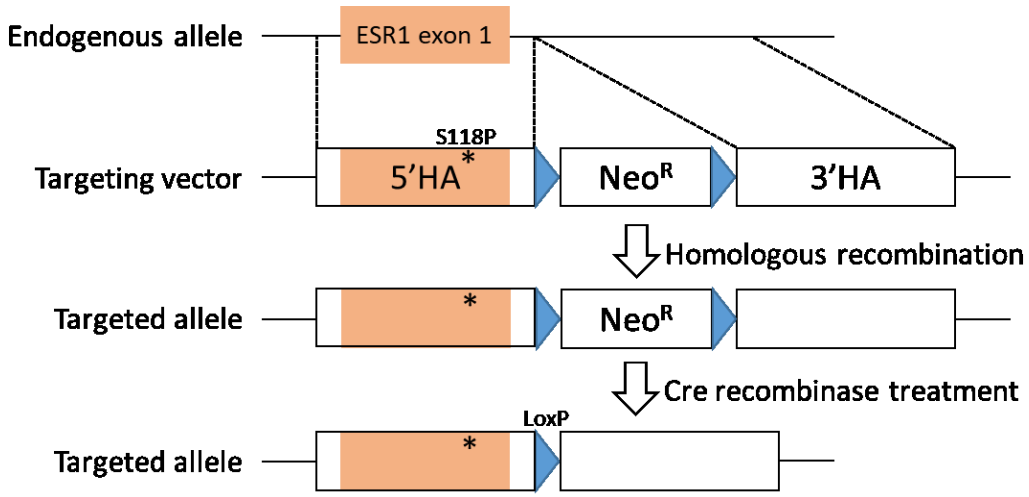

Figure 5: Recombinant AAV targeting strategy for knock-ins. In our knock-in strategy, one endogenous allele of *ESR1* undergoes homologous recombination with a recombinant targeting vector consisting of the adeno-associated viral backbone modified to contain a homologous region of *ESR1* and a neomycin resistance cassette. After selecting targeted cells with neomycin and screening for successful knock-in, the remaining cells are treated with Cre recombinase and left with a small LoxP scar between the two homologous regions.

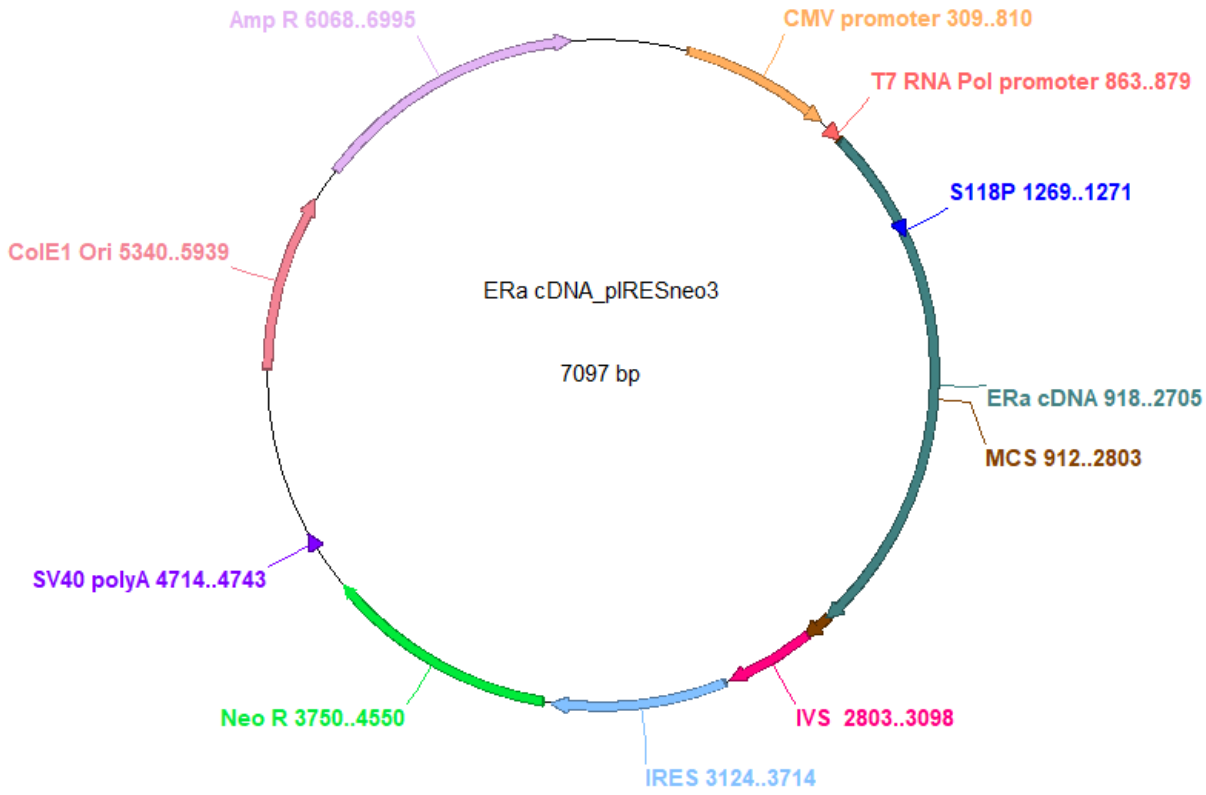

Figure 6: ER S118P overexpression vector. In our overexpression strategy, a plasmid containing the modified ER S118P cDNA was stably transfected into MCF10A cells. This vector contains an internal ribosomal entry site (IRES) followed by a neomycin resistance cassette to improve selection of cells expressing the preceding ER cDNA sequence.

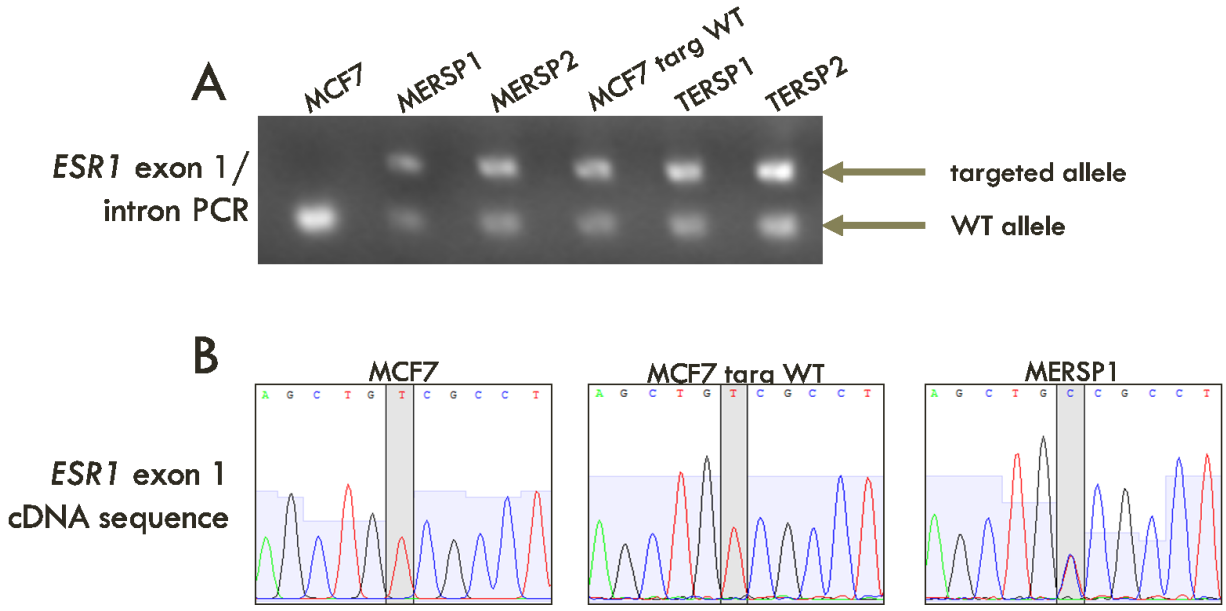

Figure 7: Validation of MERSP and TERSP clones. All cell lines were confirmed by PCR and Sanger sequencing. A) The targeted allele can be detected by PCR due to the LoxP scar within the intron immediately following *ESR1* exon 1, which results in a size difference in PCR product. B) Each cell line was additionally validated by Sanger sequencing of gDNA and cDNA.

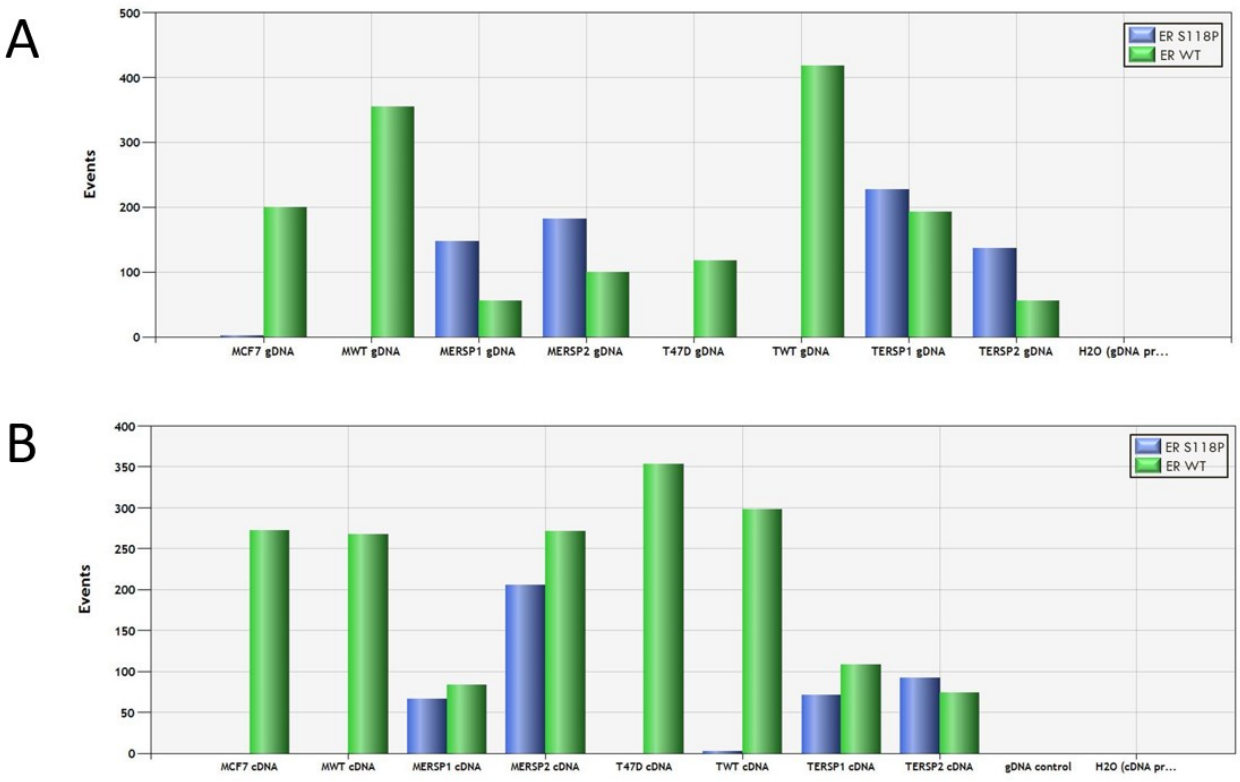

Figure 8: Allelic ratios of ER S118P cell line panel. Droplet digital PCR analysis of the MERSP and TERSP cell lines indicates that MERSP1, MERSP2, and TERSP2 have additional off-target integration of the ER S118P homology construct elsewhere in the genome. Each event represents a droplet containing an amplicon with genotype S118P (blue) or WT (green). A) Genomic DNA analysis shows that MERSP1, MERSP2, and TERSP2 have a ratio of approximately 2:1 between ER S118P and WT ER. A 1:1 ratio would be expected for a true heterozygous cell, as seen in TERSP1. B) Complementary DNA analysis shows that the additional integrant suggested in three cell lines in (A) is not expressed, as the allelic ratios of ER S118P to WT ER are close to or less than 1:1.

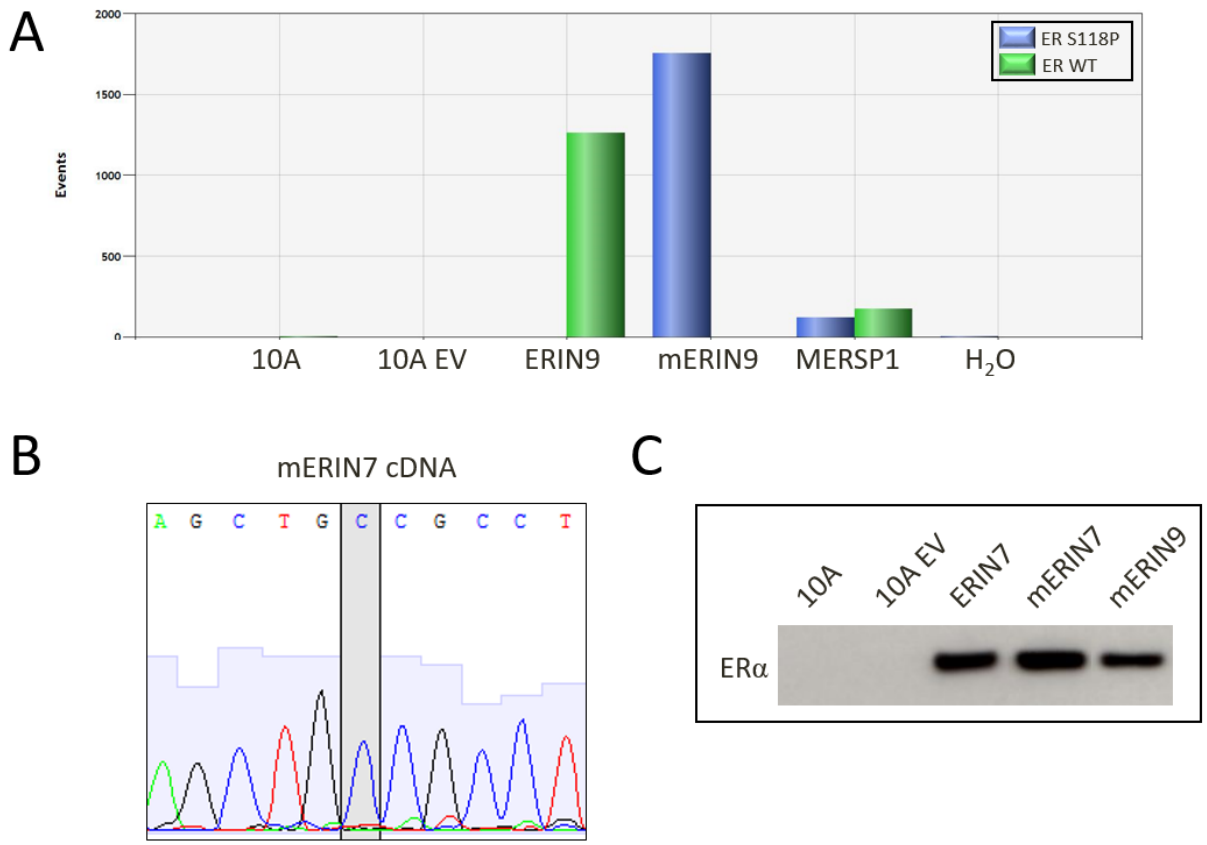

Figure 9: Validation of mERIN clones. (A) Complementary DNA analysis by droplet digital PCR shows that ERIN9 and ERIN7 (now shown) express only WT ER, while mERIN9 and mERIN7 (not shown) express only ER S118P. (B) Sanger sequencing of cDNA from mERIN7 and mERIN9 (not shown) confirm that the transcript carries the sequence resulting in ER S118P protein. (C) Western blot analysis confirms that ERIN7, mERIN7, and mERIN9 express a 66 kD ER while MCF10A and MCF10A containing the empty vector do not express ER.

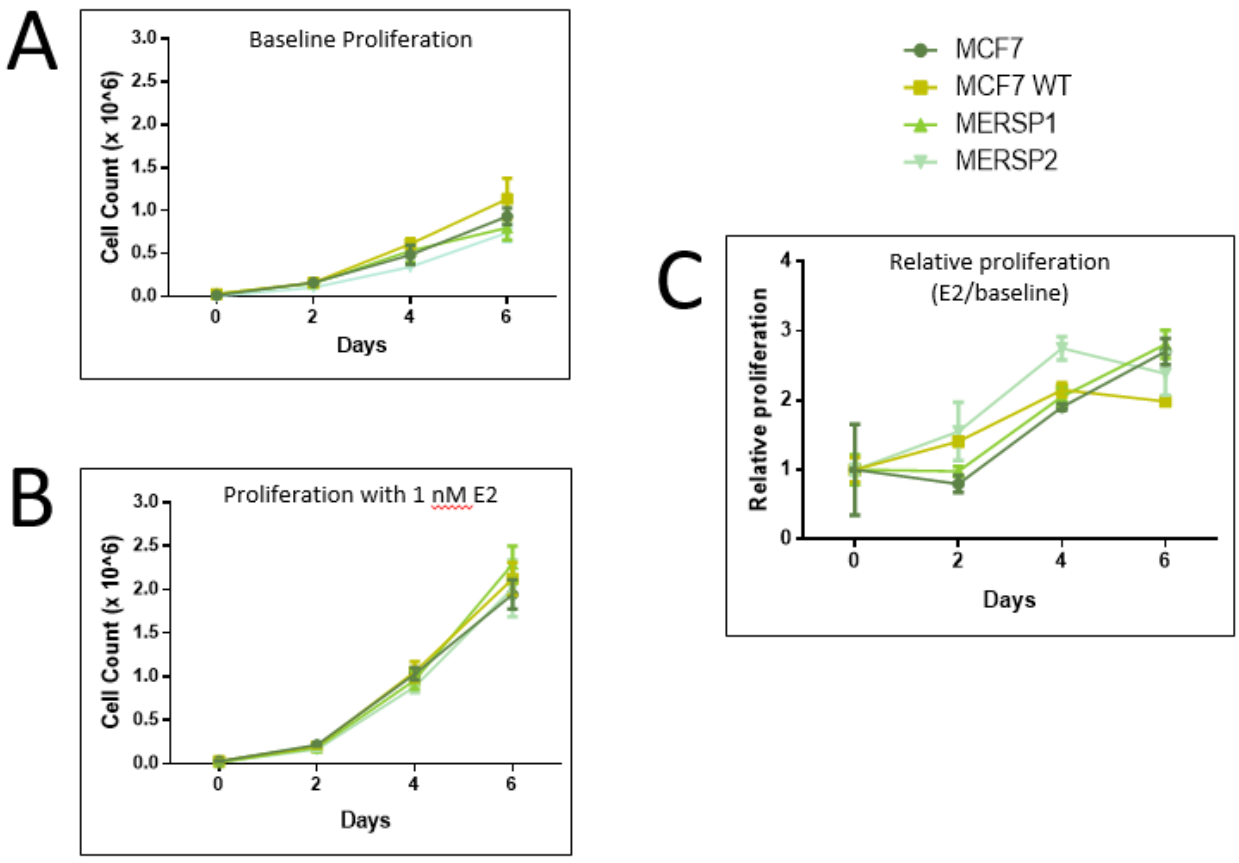

Figure 10: MCF7 and derivatives have similar growth rates in the absence and presence of 1 nM estrogen. MCF7-derived cell lines were seeded in 12 well plates in media supplemented with 5% serum and vehicle (A) or 1 nM E2 (B). Increase in proliferation in response to E2, as shown in (C) by normalizing cell counts in the presence of E2 to cell counts from baseline proliferation, remains similar between cell lines. Data are shown as mean + SD of three replicates, and curves are representative of three independent experiments.

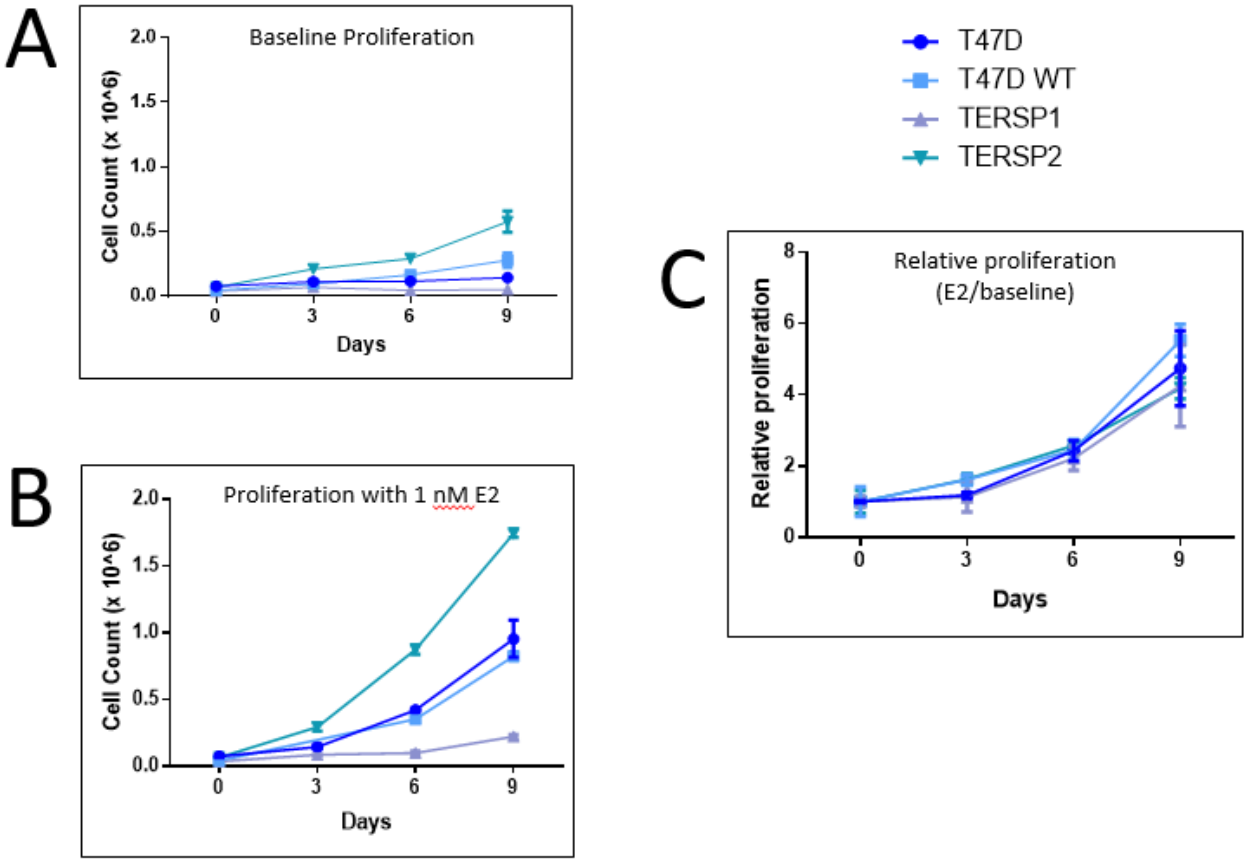

Figure 11: T47D and derivatives have variable growth rates. T47D-derived cell lines were seeded in 12 well plates in media supplemented with 5% serum and vehicle (A) or 1 nM E2 (B). In the absence and presence of 1 nM E2, TERSP1 proliferates at a slower rate than T47D and T47D WT, while TERSP2 proliferates at a faster rate. However, response to E2, as shown in (C) by normalizing cell counts in the presence of E2 to cell counts from baseline, is similar between cell lines. Data are shown as mean + SD of three replicates, and curves are representative of three independent experiments.

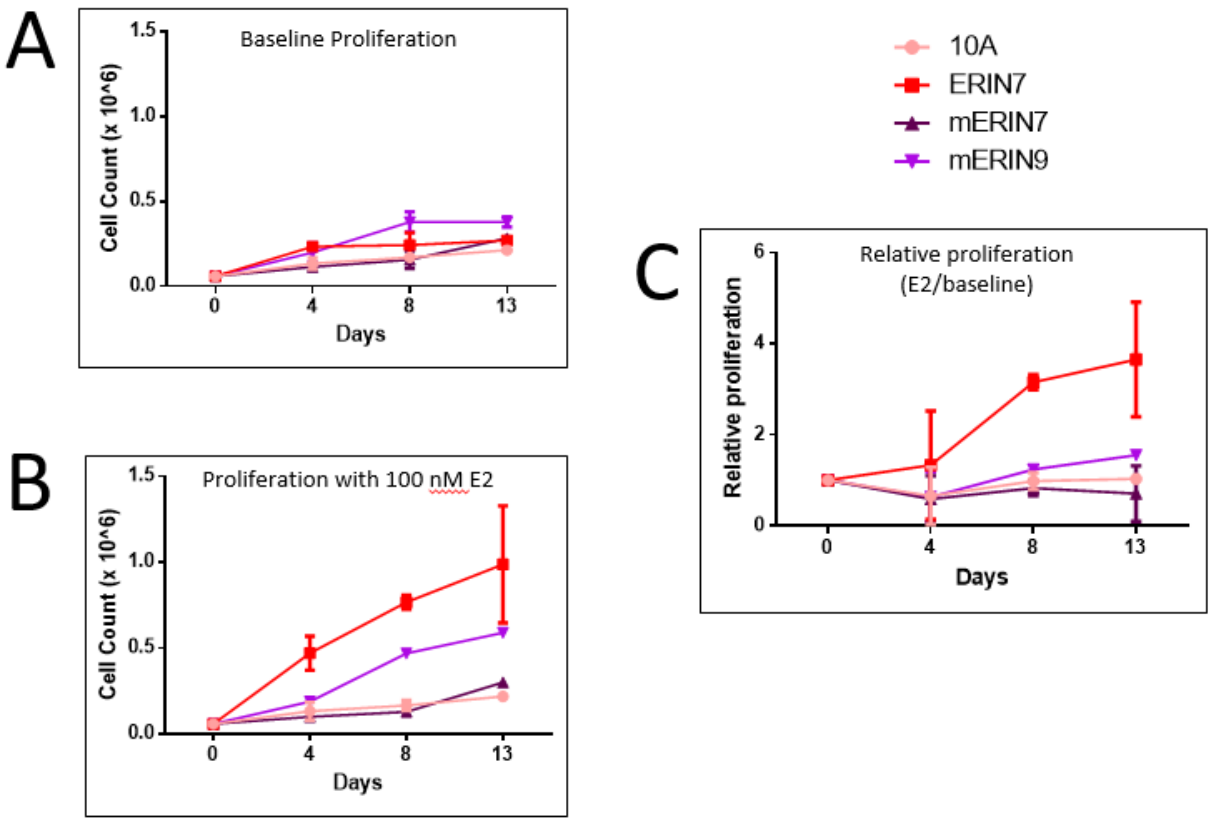

Figure 12: mERIN clones do not respond to 100 nM E2. (A) Baseline proliferation in the absence of E2 is similar between ERIN7, mERIN7, and mERIN9. (B) ERIN7 responds strongly in the presence of 100 nM E2 while mERIN7 displays a lower responses, and there is no effect on mERIN7 or the non-ER expressing MCF10A control. (C) Response to E2, shown as relative proliferation with 100nM E2 normalized to baseline proliferation, is only present in the ERIN7 cell line. Data are shown as mean + SD of three replicates, and curves are representative of three independent experiments.

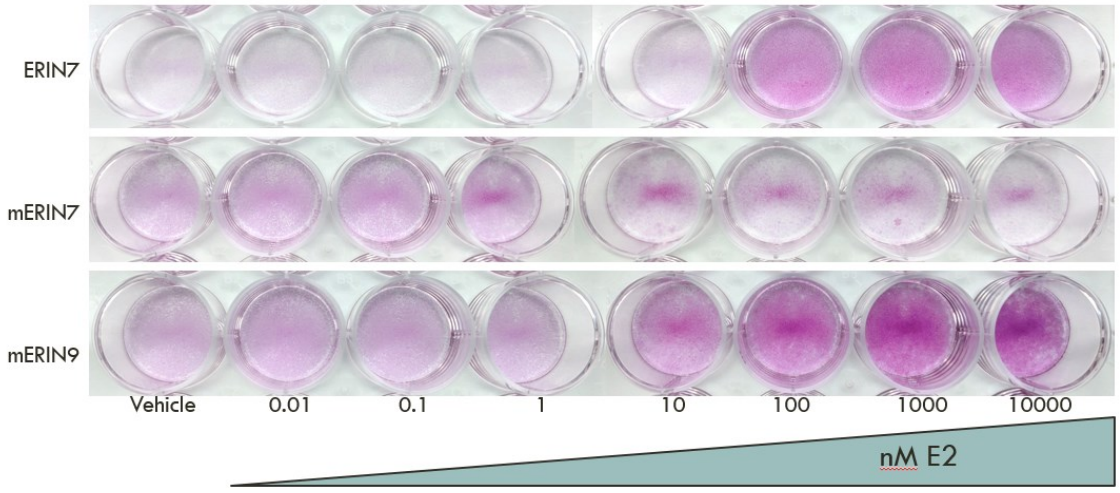

Figure 13: Additional concentrations of E2 for ERIN and mERIN clones. Additional lower concentrations of E2 reveal an increased response from the mERIN clones at 10 nM E2, which is sustained at higher concentrations in mERIN9 but not in mERIN7.

Gene Symbol	ΔΔCt MCF7 (represents E2 activation	ΔΔCt MERSP1 (represents E2 activation	(activation of MCF7 v. MERSP1)
ACTB (control)	0	0	0
AHR	0.95	-0.46	1.41
AKAP1	1.21	-0.20	1.41
B2M	0.99	-0.01	1.00
BCAR1	1.06	0.45	0.61
BCL2L1	0.89	-0.29	1.18
BDNF	0.79	-0.16	0.95
BMP4	1.22	0.67	0.55
BMP7	1.34	-0.29	1.63
BRCA1	0.79	-0.10	0.89
CAV1	1.18	0.15	1.03
CCND1	1.41	0.56	0.85
CITED2	0.86	-0.21	1.07
CKB	0.91	-0.38	1.29
CTGF	-0.36	-1.22	0.86
CTSD	2.24	1.27	0.97
CYP1A1	1.10	1.75	-0.65
EBAG9	0.85	-0.35	1.20
EFNA5	1.03	-0.25	1.28
EGR3	2.57	2.09	0.48
ERBB2	0.63	-0.42	1.05
ERBB3	0.96	-0.22	1.18
ESR1	0.79	-0.12	0.91
FOS	1.93	1.44	0.49
FOXA1	-0.08	-0.20	0.12
G6PD	1.35	0.07	1.28
GAPDH	0.85	-0.34	1.19
GPER	0.18	-0.20	0.38
GUSB	0.95	-0.36	1.31
HPRT1	0.75	-0.60	1.35
HSP90AA1	0.73	-0.21	0.94
IGFBP4	1.95	1.15	0.80
IGFBP5	1.20	0.28	0.92
IRS1	1.08	0.12	0.96
JUNB	0.55	-0.35	0.90
L1CAM	1.22	-0.53	1.75
LGALS1	1.01	-0.09	1.10
LTBP1	0.29	-0.07	0.36

Table 2: Transcriptional activation by E2 in MCF7 and MERSP1. E2-induced transcriptional activation in cells treated with 10 nM E2 for 90 minutes (as measured by comparing to cells treated with vehicle for 90 minutes) follows similar patterns in both MCF7 and MERSP1 cells.

Gene Symbol	ΔΔCt MCF7 (represents E2 activation	ΔΔCt MERSP1 (represents E2 activation	(activation of MCF7 v. MERSP1)
MAFF	2.05	0.56	1.49
MED1	1.26	-0.68	1.94
MTA1	1.08	0.13	0.95
MYC	1.80	0.86	0.94
NAB2	1.36	-0.39	1.75
NCOA1	0.95	-0.44	1.39
NCOA2	0.94	-0.35	1.29
NCOA3	1.22	-0.53	1.75
NCOR1	1.12	-0.43	1.55
NCOR2	1.21	0.07	1.14
NOV	1.02	-0.20	1.22
NR2F6	1.14	0.25	0.89
NR3C1	0.58	-0.42	1.00
NR5A2	1.58	0.44	1.14
NRIP1	2.68	1.49	1.19
NRP1	0.97	-0.42	1.39
PDZK1	0.50	0.75	-0.25
PGR	2.47	1.59	0.88
PHB2	0.83	-0.50	1.33
PTCH1	0.27	-0.49	0.76
RALA	0.76	-0.21	0.97
RARA	2.18	1.05	1.13
RPLP0	0.93	-0.03	0.96
S100A6	0.36	0.21	0.15
SAFB	0.85	0.32	0.53
SNAI1	2.31	2.02	0.29
SOCS3	3.10	2.25	0.85
TBP	0.90	-0.33	1.23
TFF1	0.87	0.71	0.16
TGFA	1.14	0.12	1.02
TGFB3	1.39	0.54	0.85
THBS1	1.74	0.73	1.01
VDR	1.84	0.47	1.37
VEGFA	1.24	0.49	0.75
WISP2	1.51	0.03	1.48
WNT4	4.24	-0.35	4.59
WNT5A	3.06	-0.81	3.87
XBP1	2.82	1.34	1.48

(Table 2 continued)

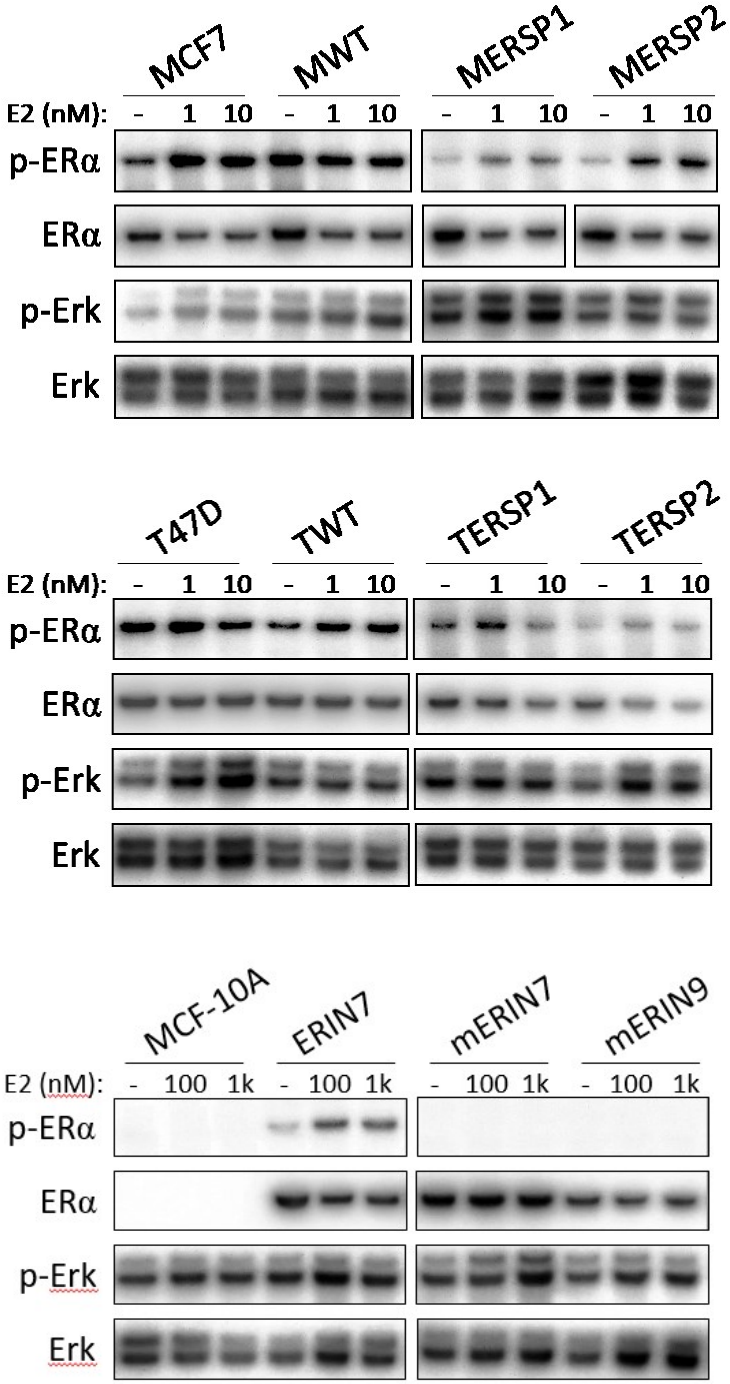

Figure 14: ER S118P cell lines maintain ER and Erk signaling patterns. After 24 hours of E2 treatment, all cell lines had reduced total ER levels and slightly increased phospho-Erk levels. Subtle variations do not correlate with ER S118P genotype. Blots are representative of three independent experiments.

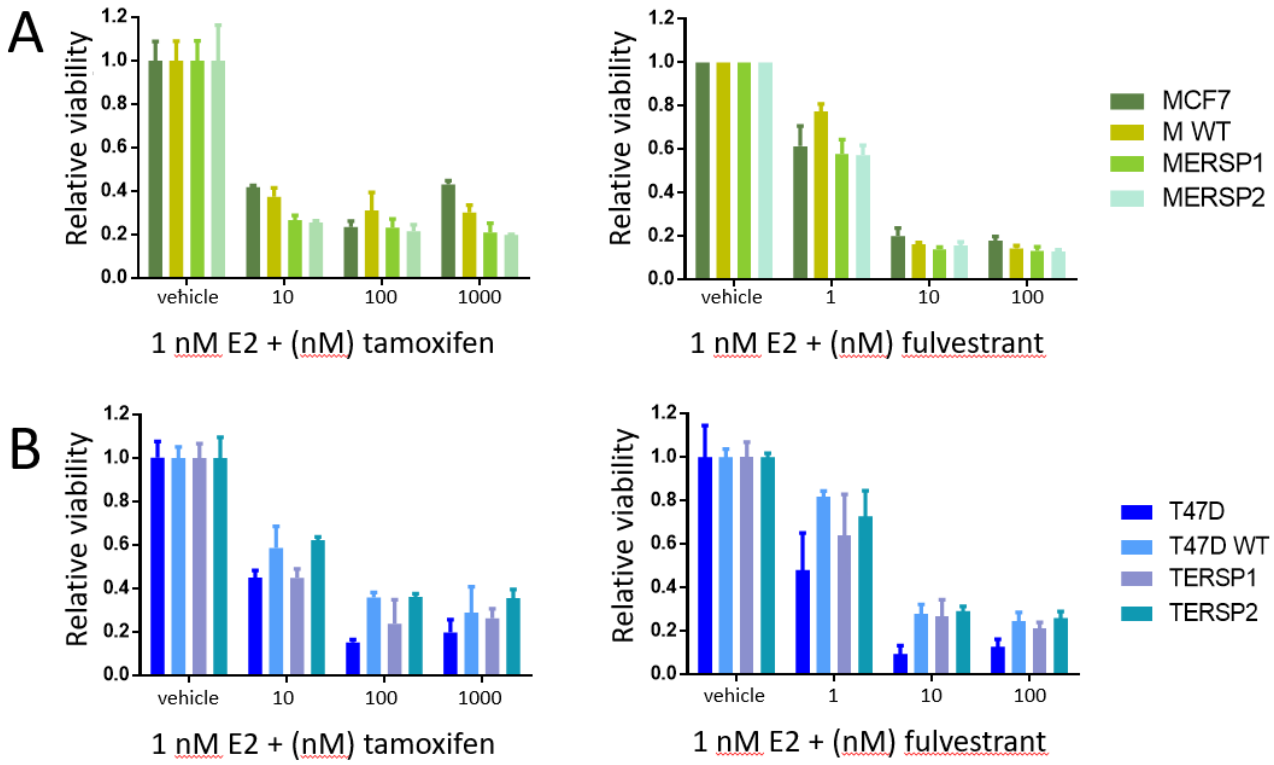

Figure 15: ER S118P cells maintain sensitivity to tamoxifen and fulvestrant. A) MCF7 cells and B) T47D cells and all targeted cell line derivatives were treated with 1 nM E2 and variable concentrations of tamoxifen and fulvestrant over nine days. Data are shown as mean + SD of three replicates relative to cells treated with 1 nM E2 + drug vehicle, and graphs are representative of three independent experiments.

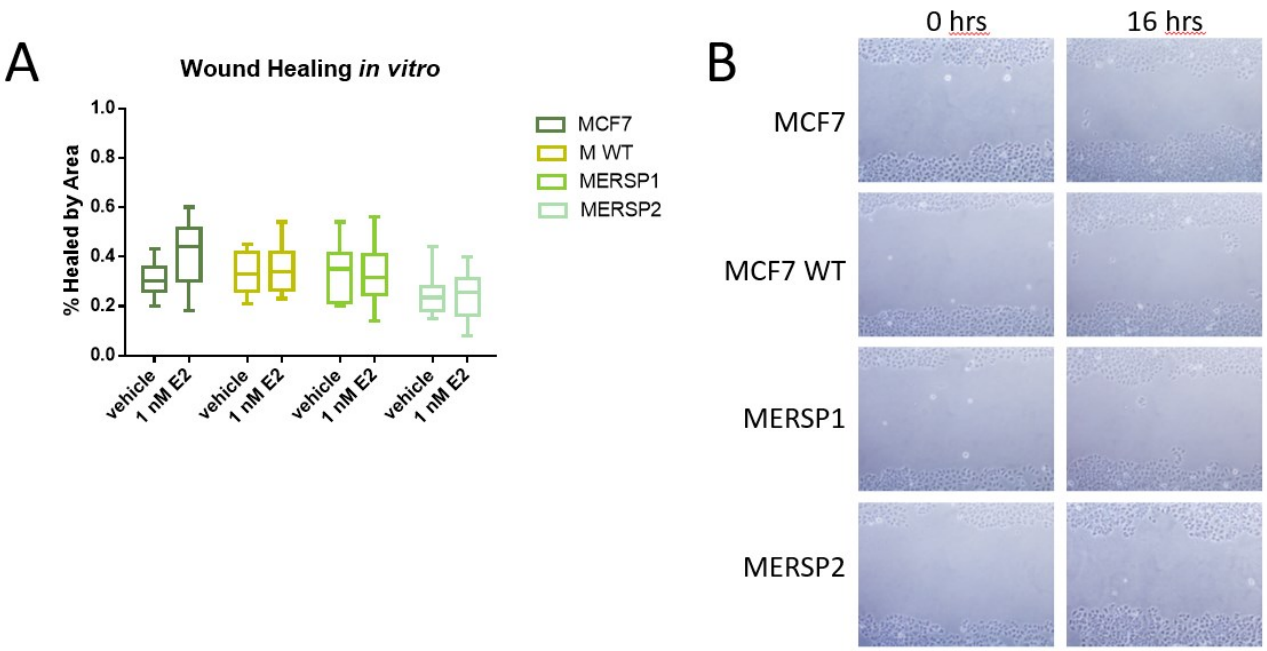

Figure 16: ER S118P does not affect cell migration toward a wound. MCF7 and targeted cell line derivatives were seeded in 6 well plates with and without 1 nM E2. Once confluent, wells were scratched with a pipet tip and monitored over 16 hours for wound closure. A) Percent healing of the wound by area was determined by subtracting cell coverage at 0 hours from cell coverage at 16 hours. B) Representative images of scratch wound healing. Data are representative of four independent experiments.

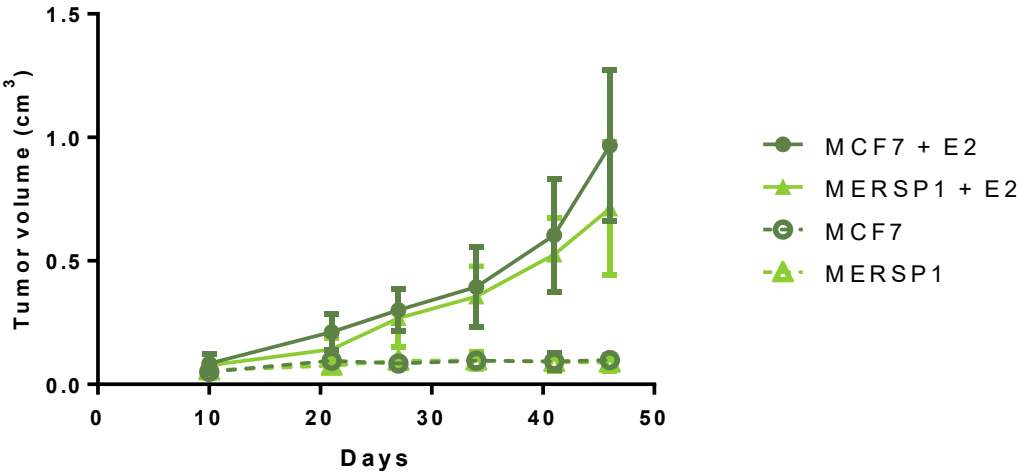

Figure 17: MCF7 and MERSP1 xenograft formation is estrogen-dependent. MCF7 and MERSP1 cells were injected into nude mice with and without estrogen supplementation implants and mice were monitored for 45 days. Both cell lines formed tumors only in the presence of estrogen. Data are shown as mean + SD of five mice per group.

Source	Population	Variant Allele Count	Total Allele Count	Allele Frequency	
Exome Aggregation Consortium (ExAC)		312	31964	.0098	*2 homozygotes
	Finnish	55	708	.0777	
	European (non-Finnish)	222	17332	.0128	*2 homozygotes
	Latino	25	2288	.0109	
	African	5	2402	.0021	
	South Asian	5	6288	.0007	
	East Asian	0	2746	0	
	Other	0	200	0	
Exome Variant Server (GO-ESP)		31	12278	.0025	*0 homozygotes
	European American	27	8156	.0033	
	African American	4	4122	.0010	
Atherosclerosis Risk in Communities		44	15726	.0028	
	European American	40	10230	.0039	
	African American	4	5496	.0007	
TOPMed		78		.0027	
1000 Genomes		8		.0016	

Table 3: ER S118P variant allele frequency in the general population. Publicly available datasets establish a minor allele frequency for this variant ranging from 0.0016 to 0.0098 (51-54).

Variable	Cancer-free (N=268)	BC survivors (N=268)
Age at baseline, years, mean (SD)	50.1 (11.2)	50.6 (11.3)
Postmenopausal, %	44.4	60.4
Age at menopause, years, mean (SD)	49.7 (6.3)	48.9 (5.4)
BMI, kg/m2, mean (SD)	27.0 (6.6)	26.2 (5.6)
Physical activity, mean MET-h/week (SD)[a]	27.4 (31.8)	26.7 (30.3)
Smoking		
Never	58.6	54.9
Former	36.2	40.3
Current	4.5	4.5
Missing	0.7	0.4
Alcohol intake, mean (SD)	6.6 (10.3)	5.8 (10.0)
Time from diagnosis to baseline, years, mean (SD)	-	2.8 (4.2)
Age at diagnosis, years, mean (SD)	-	47.8 (10.9)
Breast cancer stage, %		
In situ	-	0.7
Stage I-III	-	96.6
Stage IV	-	2.6
ER-status, %		
Negative	-	23.9
Positive	-	75
Missing	-	1.1
PR-status, %		
Negative	-	31.7
Positive	-	67.2
Missing	-	1.1
HER2 status, %		
Negative	-	81.3
Positive	-	14.6
Unknown/Missing	-	4.1
Triple negative breast cancer, %	-	18.7

[a] Metabolic equivalents from recreational and occupational activity

Table 4: BOSS cohort characteristics.

Variable	ER S118 WT (N=526)	ER S118P (N=4)
Breast cancer survivor, %	50	50
Age at baseline, years, mean (SD)	50.3 (11.2)	52.2 (13.0)
Postmenopausal, %	52.3	75
Age at menopause, years, mean (SD)	49.2 (5.8)	52.3 (9.1)
BMI, kg/m2, mean (SD)	26.6 (6.1)	26.0 (4.9)
Physical activity, MET-h/week mean (SD)[a]	27.1 (31.1)	19.3 (22.8)
Alcohol intake, mean (SD)	6.3 (10.1)	4.1 (3.9)
Smoking		
Never	57.1	0
Former	37.8	100
Current	4.5	0
Missing	<1	0

[a] Metabolic equivalents from recreational and occupational activity

Table 5: ER S118P carrier characteristics.

3 Materials and Methods

Materials and Methods

Cell culture

MCF7, T47D, and MCF10A parental cell lines were obtained from ATCC and verified via short tandem repeat profiling by the Johns Hopkins Genetic Resource Core Facility. All cells were grown in 5% CO_2 at 37° C. MCF7 and T47D lines and their derivatives were maintained in DMEM containing 5% fetal bovine serum and 1% penicillin/streptomycin. The MCF10A line and its derivatives were maintained in DMEM:F12 containing 5% horse serum, 1% pen/strep, epidermal growth factor at 20 ng/ml, insulin at 10 µg/ml, hydrocortisone at 0.5 µg/ml, and cholera toxin at 0.1 µg/ml. Cells were arrested in clear DMEM:F12 with 1% charcoal dextran treated fetal bovine serum and 1% pen/strep and assayed in clear DMEM:F12 with 10% charcoal dextran treated fetal bovine serum and

1% pen/strep (all MCF10A media contained insulin, cholera toxin, and hydrocortisone at the concentrations stated above, but EGF was omitted in arrest and assay formulations).

Gene targeting

MCF7 and T47D parental cell lines were genetically altered using a recombinant AAV vector containing the T>C single base-pair substitution in *ESR1* exon 1 resulting in ER S118P. Cells were targeted, screened, and validated as previously described (55). Two independently derived clones containing the variant and one targeted wild-type clone were isolated from each parental cell line and confirmed via gDNA and cDNA analysis by Sanger sequencing and droplet digital PCR. Targeted cells were maintained in DMEM media as described above.

Overexpression

MCF10A cells were transfected using Fugene 6 (Promega) with the pIRESneo3 vector containing a copy of ER cDNA modified with the S118P variant. Cells with stable expression of the neomycin resistance gene were selected with Geneticin (Life Technologies) at 120 ug/ml and two independently derived clones were validated for stable expression of ER S118P via cDNA ddPCR and presence of total ER but absence of phosphorylated ER S118 via immunoblotting. ERIN cell lines established previously in our lab overexpress wild-type ER from the same parent vector and were used as the wild-type control for these cells (46). ERIN and mERIN lines were maintained in DMEM:F12 media as stated above with the addition of Geneticin at 120 μg/ml.

Cell proliferation and drug response assays

17-beta estradiol, 4-hydroxytamoxifen, and fulvestrant were obtained from Sigma-Aldrich and diluted according to manufacturer recommendations. Cells were seeded at low confluency (3,000 MCF7 cells/well, 50,000 MCF10A cells/well, 60,000 T47D cells/well) in 12 well cell culture plates in arrest media for 24 hours. Cells were visualized on day 0 and estrogen/drug/vehicle conditions were added at the stated concentrations in assay media. Media containing estrogen/drug was replaced every three days. After the stated number of days, cells were trypsinized, uniformly resuspended, and counted using the Vi-Cell XR (Beckman Coulter). All results are an average of cell counts from triplicate wells.

Scratch assays

Cells were seeded at 50% confluency in 6 well cell culture plates in assay media for 24 hours. Cells were then visualized to confirm 90% confluency and placed in assay media containing 1 nM 17-beta estradiol (E2) or vehicle. After 8 hours, a 200 uL pipet tip was dragged across the plate surface, creating two perpendicular scratches in the lawn of cells; cells were then rinsed twice with clear HBSS to remove floating cell debris and media containing E2 or vehicle was replaced. Two visual fields per well were imaged at time 0 and again after 16 hours. Images were then analyzed in ImageJ using the MiToBo plugin for percent of field containing cells, and timepoints were compared in order to determine healing of the scratched area.

Immunoblotting

Cells were seeded in arrest media for 24 hours, then treated as stated. Cells were then collected and protein lysates prepared in Laemmli buffer. Lysates were analyzed by Western blot as described previously (56). Primary antibodies used in this study are: phospho-ERα S118 (Cell Signaling 2511), ERα (Cell Signaling 8644), phospho-Erk (Cell Signaling 4370), Erk (Cell Signaling 9102), GAPDH (Cell Signaling 5174).

Quantitative PCR

Cells were seeded in arrest media for 24 hours, then media was replaced with 10% CD assay media for 24 hours, then cells were exposed to 17-beta estradiol at 1 nM for 45 minutes. Cells were then trypsinized and resuspended, and RNA and cDNA were prepared. cDNA was then added to a PrimePCR pathway plate for estrogen receptor signaling (Bio-Rad) along with SsoAdvanced Universal Sybr Green Supermix (Bio-Rad) and amplified and analyzed according to the Bio-Rad protocol.

Xenografts

MCF7 cells were suspended in Geltrex LDEV-Free Reduced Growth Factor Basement Membrane Matrix (Life Technologies) and one million cells in a volume of 200 ul were injected subcutaneously into the mammary fat pad of 8- to 10-week-old female athymic nude mice (Harlan Laboratories) with and without slow-release estrogen implants as previously described (56). Tumor volumes were calculated as a pseudosphere from length, width, and height measurements. All animal experiments were performed in

accordance with institutional guidelines and The National Institutes of Health Guide for the Care and Use of Laboratory Animals guidelines.

Statistics

Statistical analyses were performed using GraphPad Prism 6 software. Relative proliferation rates were analyzed by two-way ANOVA. Error bars represent ± SD. None of the data analyzed reached a significance level of $P \leq 0.05$.

Cohort genotyping

Genomic DNA extracted from lymphocytes was obtained from Kala Visvanathan at the Johns Hopkins Center for Clinical Cancer Genetics and Prevention. Samples were analyzed via droplet digital PCR on the QX200 platform (Bio-Rad) per the manufacturer's protocols for probe-based ddPCR. Primers and probes used were as follows: forward primer (5'-CACTCAACAGCGTGTCT-3'), reverse primer (5'-CTCGTTCTCCAGGTAGTAG-3'), wild-type probe (5'-AGCTGTCGCCTTTCCTGCAG-3'), variant probe (5'-AGCTGCCGCCTTTCCTGCAG-3').

Population data

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Curriculum Vitae

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Education

Johns Hopkins University School of Medicine
Doctor of Philosophy in Cellular & Molecular Medicine (2018)

University of California, Los Angeles
Bachelor of Science in Neuroscience *Cum Laude* (2010)

Research Experience

The Johns Hopkins University School of Medicine, Breast Cancer Therapeutics/Ben Ho Park Laboratory
Graduate Student ***June 2014 – July 2018***

Characterization of the ER S118P variant in breast cancer including development of an isogenic panel of cell lines and a population study of the variant frequency

The Johns Hopkins University School of Medicine, Cellular and Molecular Medicine Program
Rotation Student ***August 2013 – June 2014***

Characterization of a DISC-1 knockdown mouse as a model of predisposition to schizophrenia and autism (Pletnikov lab); Development of high-fidelity bacterial replication technique for production of genomic tools from patient samples (Park lab); Exploration of breast cancer metastasis to brain (Riggins lab)

Translational Genomics Research Institute, Melanoma Therapeutics Lab
Research Associate ***May 2012 – July 2013***

Genetic, genomic, and epigenetic analysis of the basis of melanoma in humans and canines

Semel Institute for Neuroscience and Behavior at UCLA, Eunice Kennedy Shriver Intellectual Development and Disabilities Research Center
Undergraduate Research Assistant ***January 2008 – June 2010***

Investigation of glutamatergic response of basal ganglia neurons in mouse model of Huntington's Disease

Translational Genomics Research Institute, Neurogenomics Division, Laboratory of Dr. Kendall Jensen
Helios Scholar and Research Intern ***Summer 2009***

Development of diagnostic miRNA biosignature in mouse model of Amyotrophic Lateral Sclerosis

Translational Genomics Research Institute, Neurogenomics Division, Laboratory of Dr. Travis Dunckley

Helios Scholar and Research Intern ***Summer 2008***

Optimization of siRNA knock-down kinome screen for cell models of Parkinson's Disease with dementia

Pedagogy Training and Teaching Experience

Summer Teaching Institute, Johns Hopkins University ***Summer 2015***

Three-day workshop on evidence-based teaching methods for undergraduate instruction

An Introduction to Evidence-Based Undergraduate STEM Teaching, CIRTL Network

Online course and community for future undergraduate STEM educators ***Fall 2016***

Pollard Scholars, Johns Hopkins University School of Medicine ***Spring 2015***

Graduate program-sponsored tutor for first-year graduate students in Cellular and Molecular Medicine, selected for position according to mastery of first-year course material

Thread ***2013-2014***

Academic and life skills mentoring program to help underperforming high school students become successful in high school and college

CommunityWorks, Southwest Autism Research & Resource Center ***Fall 2011, Fall 2012***

Peer mentoring program to help teens with Autism Spectrum Disorders develop job skills

Project Brainstorm, UCLA ***Fall 2009***

Neuroscience outreach program to educate and inspire K-12 students to go to college

CalTeach, UCLA ***Spring 2008, Spring 2009***

Teaching internship at local elementary & middle schools, focus on science programs

Research Publications

Button B, Chu D, Rosen DM, Croessmann S, Zabransky DJ, Dalton WB, Cravero K, Kyker-Snowman K, Waters I, Karthikeyan S, Christenson E, Dennison L, Ramin C, May B, Roden R, Petry D, Armstrong DK, Visvanathan K, Park BH (2018). ***The estrogen receptor-alpha S118P variant does not affect breast cancer incidence or response to endocrine therapies.*** Breast Cancer Res Treat (in submission).

Cravero K, Cochran RL, Waters I, Hunter N, Christenson E, **Button B**, Kyker-Snowman K, Cole A, Canzoniero JV, Meford A, Pallavajjalla A, Park BH (2018). ***Biotinylated Amplicon Sequencing: A method for preserving DNA samples of limited quantity***. Practical Lab Med (in submission).

Avigdor BE, Beierl K, Gocke CD, Zabransky DJ, Cravero K, Kyker-Snowman K, **Button B**, Chu D, Croessmann S, Cochran RL, Connolly RM, Park BH, Wheelan SJ, Cimino-Mathews A (2017). ***Whole-exome sequencing of metaplastic breast carcinoma Indicates***

monoclonality with associated ductal carcinoma component. Clin Cancer Res 23(16):4875-4884.

Croessmann S, Wong HY, Zabransky DJ, Chu D, Rosen DM, Cidado J, Cochran RL, Dalton WB, Erlanger B, Cravero K, **Button B**, Kyker-Snowman K, Hurley PJ, Lauring J, Park BH (2017). ***PIK3CA mutations and TP53 alterations cooperate to increase cancerous phenotypes and tumor heterogeneity***. Breast Cancer Res Treat 162(3):451-464

Button B and Park BH (2016). ***ESR1 mutations: Pièce de résistance***. Genes & Diseases 3(2):124-9.

Lee J, Axilbund J, Dalton WB, Laheru D, Watkins S, Chu D, Cravero K, **Button B**, Kyker-Snowman K, Waters I, Gocke C, Lauring J, Park BH (2016). ***A polycythemia vera JAK2 mutation masquerading as a duodenal cancer mutation.*** J Natl Compr Canc Netw (in submission).

Chu D, Paoletti C, Gersch C, VanDenBerg DA, Zabransky DJ, Cochran RL, Wong HY, Toro PV, Cidado J, Croessmann S, Erlanger B, Cravero K, Kyker-Snowman K, **Button B**, Parsons HA, Dalton WB, Gillani R, Medford A, Aung K, Tokudome N, Chinnaiyan AM, Schott A, Robinson D, Jacks KS, Lauring J, Hurley PJ, Hayes DF, Rae JM, Park BH (2016). ***ESR1 mutations in circulating plasma tumor DNA from metastatic breast cancer patients.*** Clin Cancer Res 22(4):993-9.

Zabransky DJ, Yankaskas CL, Cochran RL, Wong HY, Croessmann S, Chu D, Kavuri SM, Red Brewer M, Rosen DM, Dalton WB, Cimino-Mathews A, Cravero K, **Button B**, Kyker-Snowman K, Cidado J, Erlanger B, Parsons HA, Manto KM, Bose R, Lauring J, Arteaga CL, Konstantopoulos K,Park BH (2015). ***HER2 missense mutations have distinct effects on oncogenic signaling and migration.*** Proc Natl Acad Sci USA 112(45):E6205-14.

Croessmann S, Wong HY, Zabransky DJ, Chu D, Mendonca J, Sharma A, Mohseni M, Rosen DM, Scharpf RB, Cidado J, Cochran RL, Parsons HA, Dalton WB, Erlanger B, **Button B**, Cravero K, Kyker-Snowman K, Beaver JA, Kachhap S, Hurley PJ, Lauring J, Park BH (2015). ***NDRG1 links p53 with proliferation-mediated centrosome homeostasis and genome stability.*** Proc Natl Acad Sci USA 112(37):11583-8.

Research Presentations

Cellular and Molecular Medicine Program Retreat, JH School of Medicine
"Modeling the ER S118P variant in breast cancer" ***September 14, 2017***

Breast Cancer Research Program Retreat, JH Sidney Kimmel Comprehensive Cancer Center
"Estrogen receptor variants in ER+ hormone-resistant breast cancer" ***April 21, 2017***

UCLA Neuroscience Undergraduate Poster Session
"Altered GABAergic function in striatal large cholinergic interneurons in the R6/2 model of Huntington's Disease" ***May 27, 2010***

Helios Scholars Annual Symposium Poster Session ***July 31, 2009***

"A diagnostic for Amyotrophic Lateral Sclerosis: Finding a microRNA signature"

Helios Scholars Annual Symposium Poster Session ***July 30, 2008***
"The pathology of Parkinson's Disease with dementia: Phosphorylation of α-synuclein"

Awards and Honors

Outstanding Poster, Helios Scholars Symposium ***July 2009***

Helios Scholar, Translational Genomics Research Institute ***2008 and 2009***
Selected out of hundreds of applicants for an internship class of 40 students

Latin Honors at UCLA ***2007-2010***
Graduated *cum laude* from UCLA in three years